Reprinted from the American Journal of Physiology.

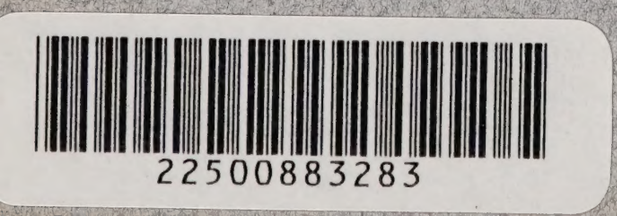
Vol. XXXI. — October 1, 1912. — No. I.

THE NATURE AND ACTION OF THE THROMBOPLASTIC (ZYMOPLASTIC) SUBSTANCE OF THE TISSUES.

By W. H. HOWELL.

[From the Physiological Laboratory of the Johns Hopkins University.]

WELLCOME INSTITUTE LIBRARY
Coll. wellcOmec
Coll. pam.
No. WH 310
1912
H85n



Reprinted from the American Journal of Physiology.
Vol. XXXI. — October 1, 1912. — No. I.

THE NATURE AND ACTION OF THE THROMBOPLASTIC (ZYMOPLASTIC) SUBSTANCE OF THE TISSUES.

By W. H. HOWELL.

[*From the Physiological Laboratory of the Johns Hopkins University.*]

IT has long been known that the coagulation of blood is hastened by contact with the tissues. This fact has been demonstrated by Arthus[1] and others for the mammalian blood, but the accelerating effect of the tissues is shown most strikingly in the case of those bloods whose normal, so to speak, intrinsic coagulation occurs very slowly. Such bloods are especially the blood of the birds when removed carefully from the blood vessels so as not to come into contact with the tissues, and the so-called peptonized blood of the dog. Bloods of this kind may be made to clot promptly by the addition of saline or aqueous extracts of various tissues, thymus gland, muscle, brain, testis, etc. The extractible substance or substances in the tissues which facilitate the process of clotting, which indeed under normal conditions are essential to the prompt clotting of the blood, have been designated as zymoplastic (Schmidt) or thromboplastic (Nolf) substances, although other names of a more specific character have been suggested. The explanation of the manner of action of these substances has varied naturally with the views adopted regarding the nature of the processes of coagulation. According to some authors (Pekelharing, Loeb, *et al.*), they are substances identical with or similar in action to the fibrin ferment (thrombin). According to Arthus, they are substances of an unknown nature which accelerate the

[1] Arthus: Journal de physiologie et de pathologie, 1902, iv, p. 281.

formation of thrombin by action upon the leucocytes. Bang [2] has suggested a somewhat similar view, namely, that the thromboplastic substances are lipoids which stimulate or activate the tissue cells to produce thrombokinase. Both of these views which attribute to the thromboplastic substance a stimulating action upon formed elements would seem to be contradicted conclusively by Hewlett's experiments. This author [3] showed that the cell-free plasma of bird's blood is made to clot by the addition of tissue extracts, and a similar result may be demonstrated easily for cell-free peptone plasmas. Schmidt explained the action of the thromboplastic material on the view that they cause the formation of thrombin from prothrombin by a process of splitting, or, as we might say now in more general terms, by a process of activation. An essentially similar hypothesis, although expressed in different terms, has been advanced by Morawitz [4] and by Fuld and Spiro.[5] They assume that the thromboplastic substance is of the nature of an activator or co-ferment which is essential to the conversion of prothrombin (thrombogen) to active thrombin. Morawitz designated the substance as a kinase (thrombokinase), while Fuld and Spiro described it under the name cytozym. In Nolf's [6] somewhat intricate theory of coagulation the favoring action of thromboplastic agents is explained on the view that they act as centres of precipitation in the colloidal interaction between fibrinogen, thrombogen, and thrombozym. In contrast to these theories the author [7] has furnished evidence to show that the thromboplastic material in tissue extracts facilitates clotting only in those plasmas in which antithrombin is present and that it acts by neutralizing the antithrombin.

It is easily proved and is perhaps generally admitted that avian blood and the blood of a peptonized dog owe their relative incoagulability to antithrombin, the presence of which can be detected and the amount estimated relatively by the use of test solutions contain-

[2] Bang: Ergebnisse der Physiologie, 1909, viii, p. 476.

[3] Hewlett: Archiv für experimentelle Pathologie und Pharmakologie, xlix, p. 307.

[4] Morawitz: Hofmeister's Beitrage zur chemische Physiologie und Pathologie, 1904, iv, p. 381; also Ergebnisse der Physiologie, 1905, iv, p. 405.

[5] Fuld and Spiro: *Ibid.*, 1904, v, p. 174.

[6] Nolf: Archives internationales de physiologie, 1908, vi, pp. 1 and 115; also Revue générale des sciences pures et appliquées, 1909, July 15.

[7] Howell: this Journal, 1911, xxix, p. 187.

ing definite amounts of thrombin and fibrinogen. These bloods can be made to clot by the direct addition of thrombin solutions provided enough is added to overcome the excess of antithrombin present. The older statement of some authors that peptonized blood does not clot upon the addition of fibrin ferment (thrombin) is entirely erroneous and due to the fact that in their experiments too little thrombin was used. On the other hand, peptonized plasma is clotted very readily by the addition of tissue extracts. In this case what takes place is that the thromboplastic substance in the extracts neutralizes the antithrombin, and thus permits the thrombin already present in the blood to effect its normal action upon the fibrinogen. The author [8] has shown that antithrombin in small amounts is a constant constituent of mammalian blood, and that the facilitation of the clotting of this blood caused by tissue extracts, or by simple contact with the tissues, is due to the fact that the antithrombin is neutralized by the thromboplastic substance in the tissues. Further evidence in favor of this explanation of the action of thromboplastic substance is furnished in this paper.

Much uncertainty has prevailed in regard to the chemical nature of the thromboplastic substance. It has been especially confusing to find that some authors have stated that this substance is water-soluble only and that its activity is destroyed by heating, while others have claimed that it may be extracted from the tissues with alcohol or ether and that its action is not destroyed by boiling. It would seem at first, from these and other contradictory statements, that there must be a variety of thromboplastic substances. As will be shown, however, in this paper, these contradictory statements may be reconciled, and there is every reason to believe that the thromboplastic substance is essentially the same in all tissues and acts in all cases in the same way.

Thromboplastic solutions are made usually by extracting a minced tissue with water or a saline solution. The extract should be freed from suspended particles by repeated filtration or by centrifugalization. As a rule, the extracts are very opalescent, but they may be obtained nearly clear by repeated filtration while still very active. The most successful perhaps of the efforts made to isolate further the active substance in these extracts is that reported by Wooldridge.

[8] Howell: *Loc. cit.*

Wooldridge himself did not accept the usual theory of coagulation, that is to say, a reaction between fibrinogen and thrombin, and he did not make use of the term thromboplastic or zymoplastic substance. There can be little doubt, however, that his A-fibrinogen of the blood, or his tissue fibrinogen obtained from other tissues, is identical with what other authors have designated as thromboplastic or zymoplastic substance. In 1886 [9] he prepared this substance from the thymus gland by the following method. Calf's thymus was extracted with water and the extract was centrifugalized. The solution was then made strongly acid with acetic acid, and the voluminous precipitate obtained was washed with water and then dissolved in a dilute solution of sodium carbonate. He showed that such solutions cause intravascular clotting when injected into the circulation, that they have no direct thrombin action as tested upon magnesium-sulphate plasmas, and that the active constituent may be extracted from thymus tissue with alcohol and ether. In recent years the same method substantially has been described by Horneffer[10] and Battelli [11] for the preparation of what they designate as pure thrombokinase. The substance precipitated by the acetic acid is evidently a protein, but whether or not its undoubted activity in facilitating the process of clotting is due to the protein or to some accompanying substance was not determined. Schmidt long ago claimed that an active zymoplastic substance can be extracted from the tissues with alcohol and ether, and he suggested that possibly this substance is lecithin. Wooldridge also found that alcohol-ether extracts of red or white blood corpuscles or of other tissues yielded a substance corresponding with what was then known as lecithin, which had the same action in clotting peptone plasmas as aqueous extracts of the tissues. He stated, however, that lecithin prepared from the yolk of egg has no such action. This curious difference indicated that the active substance in the alcohol-ether extract of the tissues is either something else than lecithin, or that the lecithin of the tissues varies in some way from the stored lecithin of the egg yolk. While Wooldridge did not identify the active material in thromboplastic solutions as lecithin, since the whole conception of thromboplastic activity was excluded from his theory,

[9] Wooldridge: Archiv für Physiologie, 1886, p. 397.

[10] Horneffer: Thèse de Genève, 1908.

[11] Battelli: Comptes rendus de la Société de Biologie, 1910, May 7.

yet, as a matter of fact, with our present knowledge we must interpret his results, together with those of Schmidt, as pointing to the belief that a lecithin is the active constituent in thromboplastic extracts. The present paper gives an account of experiments and observations the end result of which has been to strengthen this conclusion and to reconcile it with the apparently contradictory results reported by other authors.

Preparation of the Peptone Plasma.

In experiments upon the thromboplastic activity of tissue extracts it is necessary above all things to have a suitable solution upon which the facilitating action of these extracts may be tested. The centrifugalized plasma of a peptonized dog furnishes such a solution, but, as is well known, it is somewhat difficult to prepare such plasmas so that they shall have a constant coagulability. According to the dose and the character of the peptone used and the time after peptonization at which the blood is drawn the plasma may contain little or much antithrombin and will show varying degrees of coagulability. Even when all the conditions are kept as constant as possible, the effect of the injection, so far as the coagulability of the drawn blood is concerned, may vary with the animal used. This difficulty has been avoided in these experiments by preparing a large amount of clear peptone plasma from one dog and drying it down to make a stock material. To do this the plasma was evaporated to dryness in watch crystals, each containing 2 c.c. of the plasma, placed in a current of warm air but with precautions to keep the temperature below 40° C. The watch crystals containing the dried plasma were kept in a desiccator, and when a specimen of peptone plasma was needed the material in one of the crystals was rubbed up with 4 c.c. of a 0.9 per cent solution of sodium chloride and filtered. The plasma thus obtained may be used directly or, in order to increase its coagulability, and therefore its sensitiveness as a reagent for the detection of thromboplastic substance, it may be diluted with an equal volume of water. The peptone plasma originally used was obtained by injecting a solution of Witte's peptone of known efficiency into a dog in the proportion of 0.4 to 0.5 gm. to each kilogram of weight of the animal. The dog was anesthetized with morphia and ether, and the whole amount of peptone

used was forced quickly into the circulation by injection into the femoral artery under pressure. In order that the plasma may be free from fat it is desirable that the animal should have fasted for twenty-four hours. Twenty minutes after the injection the animal was bled to death, the blood was centrifugalized at once, and the clear plasma was evaporated to dryness in watch crystals, as described above. In this way material was obtained for a series of experiments whose coagulability toward thromboplastic solutions was quite uniform. It may be stated that the peptonized plasma used did not clot spontaneously in twenty-four hours, even when diluted with two to three times its volume of water. The addition of water to the saline solution of dried peptone plasma greatly increases its sensitiveness toward thromboplastic solutions, as may be illustrated by the following experiment. The thromboplastic solution used in this case was a saline extract of brain made by treating 1 gm. of dried brain with 100 c.c. of saline solution (0.9 per cent sodium chloride) and centrifugalizing.

1. Peptone plasma, five drops; water, twenty drops. No clot in twenty-four hours.
2. Peptone plasma, five drops; brain extract, five drops. No clot in twenty-four hours.
3. Peptone plasma, five drops; water, five drops; brain extract, five drops. Solid clot in seven minutes.
4. Peptone plasma, five drops; water, ten drops; brain extract, five drops. Solid clot in five minutes.

This result was constant and agrees with the observation previously reported by the author upon the effect of diluting the slowly coagulating centrifugalized plasma of the terrapin with water and with saline solution respectively. It would seem that, in plasmas containing sufficient antithrombin to retard or to prevent spontaneous coagulation, dilution with approximately isotonic solutions of sodium chloride does not alter the coagulability of the plasma, while dilution with water increases the rapidity with which spontaneous coagulation occurs in solutions containing little antithrombin, or increases greatly the sensitiveness toward the coagulating effect of thromboplastic solutions in plasmas containing much antithrombin. Dilution with water, in other words, weakens the action of antithrombin. A satisfactory explanation of this fact cannot be given until further experiments are made.

Preparation of the Thromboplastic Solutions.

In preparing thromboplastic solutions from the various tissues it was found most convenient to start with the dried tissue. The organ selected, usually the brain or the thymus gland, was washed as free as possible from blood and was then ground to a thin pulp in a mortar. This material was spread in thin layers on glass plates and was dried at a low temperature in a current of warm air. The dry material was kept in a desiccator until needed.

Ether extracts. — The thromboplastic material may be obtained from the dried tissue by extraction with alcohol, ether, or chloroform. Ether was found to be most satisfactory, and the results obtained with this solvent will be described in detail. The dried tissue, preferably the brain, was shaken with a large volume of ether and allowed to stand for some hours. The red-colored solution was filtered once or twice, until clear, and was then evaporated to dryness in a current of warm air. The soft oily reddish residue when treated with water gave a turbid solution which had a marked thromboplastic action on peptone plasma. This residue was further treated as follows. It was first extracted several times with large volumes of cold acetone and finally with hot acetone. This solvent removed the fats, cholesterin, and cholesterin esters. The first acetone extract was rich in cholesterin and cholesterin esters, and when evaporated to dryness and taken up with water in the form of an emulsion showed no thromboplastic effect at all upon peptone plasma. The later acetone extracts and particularly the hot acetone extract yielded a small residue which upon treatment with water gave a solution that exhibited a feeble but distinct thromboplastic action on peptone plasma. The results of the acetone extraction show that the thromboplastic substance is not cholesterin or a cholesterin ester. Some previous observations had led me to suspect that the active substance might be one of the cholesterin esters, but the procedure here described proves that this suspicion was not correct, and this conclusion was verified by experiments with cholesteryl oleate, prepared synthetically, which showed that this substance in aqueous suspension is entirely inactive toward peptone plasmas. It is evident, however, that the active substance is slightly soluble in the acetone, especially in the hot acetone.

The residue left after the treatment with acetone had a waxy con-

sistency and was strongly thromboplastic in aqueous solution. It was treated next with a large bulk of cold alcohol. The alcoholic extract evaporated to dryness yielded a considerable residue containing a large proportion of lecithin. This residue treated with water gave an opalescent solution which had a distinct thromboplastic action, while the portion not soluble in cold alcohol when treated with water gave likewise an opalescent solution possessing marked thromboplastic action. The portion soluble in cold alcohol was evaporated to dryness, the residue was dissolved in ether, and the latter solution was precipitated with acetone. This process was repeated, and the second acetone precipitate was dissolved with some difficulty in warm alcohol; evaporated to dryness and treated with water, solutions were obtained showing thromboplastic action. It is evident that the active substance is soluble to some extent in cold alcohol, but its solubility in this solvent must be small, since a relatively large volume of alcohol was used and most of the active substance remained behind. The material remaining after extraction with cold alcohol was next treated repeatedly with large volumes of boiling alcohol. Each such treatment dissolved a part of the reddish residue, and after four or five extractions the small residue finally remaining ceased to exhibit thromboplastic action when treated with water and tested upon a peptone plasma. Each of the extracts with hot alcohol, especially the first one, gave on evaporation a reddish waxy deposit which when rubbed up with water dissolved easily to form an opalescent solution possessing marked thromboplastic action. It would appear from these results that the active substance is soluble with difficulty in hot alcohol. The residues obtained by evaporating the cold and hot alcoholic extracts consisted mainly of lecithin and related phosphatids. They contained phosphorus in considerable amounts and gave the usual myelin figures upon treatment with water.

The question of most interest is to determine whether the thromboplastic substance is lecithin itself or one of the related phosphatids, or whether perhaps this property is possessed by all the substances belonging to this group. Owing to the confusion that exists in regard to the chemistry of these bodies, it is not possible to answer this question with entire satisfaction. The chemical individuality of the several phosphatids described is not well established, and, as is well known, there is no satisfactory method of obtaining pure lecithin from the

brain or other tissues. The results of the numerous efforts made to isolate the active thromboplastic substance as found in the ether extracts of brain or thymus have led me to believe that this substance is not lecithin as usually defined, but rather the related unsaturated phosphatid, kephalin, or else, although this suggestion seems highly improbable, some unknown substance which adheres to the kephalin fraction of the phosphatid material. The reasons for this conclusion rest upon such reactions as the following: an ether extract of the dried brain was evaporated to dryness, and the residue was extracted thoroughly with acetone to remove the fats, cholesterin, and cholesterin esters. The residue remaining after the acetone extraction was dissolved in ether and precipitated by an excess of alcohol. The precipitate was separated by centrifugalizing, was dissolved in ether, and again precipitated by excess of alcohol. Since lecithin as usually described is not precipitated from its ethereal solutions by addition of alcohol, the final precipitate obtained by this treatment might be expected to consist of kephalin or of kephalin and myelin. This material in aqueous solution exhibited thromboplastic action. When treated with ether, it dissolved readily, although the solution was somewhat turbid. The ethereal solution was evaporated to dryness, and the residue was treated with a large bulk of hot alcohol. Only a portion of the residue dissolved, as would be expected from the known small solubility of kephalin even in hot alcohol. The alcoholic solution while hot was precipitated by the addition of a hot alcoholic solution of lead acetate with ammonia. An abundant precipitate was obtained, which when cold dissolved readily in ether. The ready solubility of this precipitate in ether as well as the rapid solution of the previous alcoholic precipitate in ether would indicate that no myelin was present, while, on the other hand, the solubility relations serve to identify the phosphatid as a kephalin. We must believe, therefore, that the thromboplastic action of brain and thymus tissue, and probably of all tissues, is due in part at least to the presence of a phosphatid corresponding in its properties to kephalin.

Whether or not lecithin possesses a similar property is difficult to determine. If we accept the view that brain lecithin is identical chemically with the lecithin found in the yolk of the egg, then the facts indicate that this phosphatid is without thromboplastic action. Wooldridge, as stated above, called attention to the fact that lecithin pre-

pared from the egg has no effect upon the clotting of peptone plasma, while that obtained from the tissues accelerates the clotting after the manner of tissue extracts, and in this connection it is suggestive that Koch in his investigations came to the conclusion that kephalin, as described by him, does not occur in the egg, "which consists mostly of stored material," but can be obtained only from the substance of living cells. As will be shown below, this latter statement is too sweeping, but it is very easy to show that lecithin as prepared from the egg, is entirely lacking in thromboplastic action. In demonstrating this fact I have used specimens of lecithin freshly prepared from the egg, as also several specimens which I owe to the kindness of Dr. Kyes of the University of Chicago. These latter specimens were labelled egg lecithin, Agfa, egg lecithin, Blattmann, and egg lecithin, Riedel. Each of these preparations when treated with water gave a turbid solution or emulsion, which, when added, in equal volume, to solutions of my dried peptone plasma, gave no indications of clotting after a stand of twenty-four hours. Parallel preparations made with the above-described extracts (kephalin) from the brain or thymus caused clotting in twenty to thirty minutes. A specimen of plant lecithin (Blattmann) and of so-called brain lecithin, also furnished by Dr. Kyes, possessed a distinct thromboplastic action when used in aqueous solutions. Similar negative results were obtained from the egg lecithins after they had been purified by acetone precipitation from ethereal solutions. In making fresh preparations of lecithin from the egg the same procedure was followed as in the case of the brain; that is to say, the yolks were rubbed in a mortar, spread in thin layers on glass plates, and dried in a current of warm air. The dried material was powdered and extracted with ether. The yellow ethereal solution was evaporated to dryness and extracted thoroughly with acetone. The waxy material remaining consisted chiefly of lecithin. When treated with water, it gave an opalescent solution which, tested upon specimens of peptone plasma, showed a very feeble indication of thromboplastic action. After a number of hours there might be formed an imperfect membranous clot at the bottom of the tubes. The waxy residue was further treated with cold alcohol; most of it dissolved, and the small portion remaining was only partly soluble or difficultly soluble in hot alcohol. The portion soluble in cold alcohol and the insoluble portion were treated separately for their thromboplastic action. The

former, which consisted of the lecithin proper, when brought into aqueous solution, gave wholly negative results with the peptone plasma. The portion insoluble in cold alcohol was further purified by solution in ether and precipitation with acetone. In aqueous solution it had a distinct thromboplastic effect upon the peptone plasma. It would seem from these observations that there is contained in the egg yolk a small portion of phosphatid material possessing the solubility relations of kephalin, and this material has a thromboplastic action, while the egg lecithin is entirely devoid of this property. Stern and Thierfelder [12] also report that they have prepared from egg yolk a kephalin-like body which is only slightly soluble in hot alcohol.

It was stated above that a specimen of plant lecithin, unlike the egg lecithins, showed distinct thromboplastic action. Closer examination showed, however, that the active substance in this specimen was again a kephalin-like body. The specimen was dissolved in ether and the solution was precipitated by acetone. The precipitate was treated successively with cold and hot alcohol. It was found that a portion of the precipitate was soluble in cold alcohol, a portion was soluble in hot alcohol, and a portion was insoluble or soluble with great difficulty in hot alcohol. The three portions were tested separately for their thromboplastic action. This action was quite distinct only for the part soluble in hot alcohol but insoluble in cold alcohol. Since the lecithin proper was found chiefly at least in the portion soluble in the cold alcohol, the negative reaction from it would again indicate that the lecithin does not possess thromboplastic action.

Although it is perfectly clear that egg lecithin does not possess thromboplastic properties, it still remains uncertain whether or not this statement can be made positively for the brain lecithin. The kephalin of the brain tissue is undoubtedly thromboplastic, but my experience indicates that the active phosphatid in the brain is more easily soluble in alcohol than would be expected from current descriptions of the properties of kephalin. On this latter point, however, there seems to be considerable difference of opinion, the kephalin in fact being an insufficiently characterized body. The method of separation between lecithin and kephalin by treatment with cold alcohol is unsatisfactory, as appears from results such as the following. The

[12] Stern and Thierfelder: Zeitschrift für physiologische Chemie, 1907, liii, p. 378.

dried brain was extracted with ether, the ethereal solution was evaporated to dryness, and the residue was extracted thoroughly with acetone. The portion remaining was redissolved in ether and precipitated by the addition of alcohol. Examination showed that thromboplastic substance was present in the precipitate, which contained kephalin, as well as in the filtrate, which contained the lecithin. The filtrate was evaporated to dryness, the residue was dissolved in ether and precipitated by addition of acetone. The precipitate obtained when treated with cold alcohol dissolved for the most part, and the material in solution on examination showed distinct thromboplastic action. The alcoholic solution was evaporated to dryness, and the residue was taken up with hot acetic ether. On cooling, the solution gave a white deposit which showed thromboplastic action. This material consisted for the most part certainly of lecithin, but it remains possible that enough kephalin or related phosphatid passed into solution in the alcohol to explain the thromboplastic effect. Under the circumstances it is not possible to state definitely whether or not brain lecithin has thromboplastic action, but it would seem probable that it has not, considering the clearly negative results yielded by egg lecithin.

Saline and aqueous extracts. — In making saline or aqueous extracts 1 gm. of the dried tissue was rubbed up with 100 c.c. of water or of a 0.9 per cent solution of sodium chloride. The very turbid solution obtained was centrifugalized or filtered until as clear as possible. In the case of the brain extracts made with water centrifugalization at 3000 gives an opalescent solution, and the sediment, if again treated with water and centrifugalized, yields also a turbid solution showing thromboplastic action. If the process of treating the sediment with water and centrifugalizing is continued, solutions of decreasing opacity and decreasing thromboplastic action are obtained. It is evident that in the dried brain tissue the active material is only slightly soluble in water, but by repeated extraction it may be dissolved out completely. The solutions, particularly the later clearer ones which are entirely free from suspended particles, give no visible coagulum on boiling and none of the usual protein reactions. On the contrary, the aqueous and saline extracts of the dried thymus gland and the saline extracts of the dried brain contain a protein material with which the active thromboplastic substance is combined. This protein is more abundant in the extracts from the thymus than in those from the brain. As stated

by Wooldridge, the addition of acetic acid throws down a protein material which may be redissolved in dilute solutions of sodium carbonate (thymus) and which has a strong thromboplastic action. When the saline extracts of the thymus are heated to 56° to 60° C., an abundant coagulum forms containing all the protein material. The filtrate from this precipitate has lost entirely its thromboplastic property. The most interesting fact in view of the preceding evidence in regard to the phosphatid nature of the thromboplastic substance is that when either the acetic acid precipitate or the heat coagulum is extracted with alcohol-ether an active thromboplastic substance is found in the extract. The ether or alcohol-ether extract evaporated to dryness leaves a residue which when rubbed up with water gives an opalescent solution possessing thromboplastic properties. This latter solution may be boiled without affecting its thromboplastic activity. We must conclude that in the brain and thymus, and no doubt in the tissues generally, the thromboplastic phosphatid material is combined with a tissue protein having a low temperature of heat coagulation. This protein corresponds with what Wooldridge called tissue fibrinogen, and as he suggests it may be classified in the lecithin protein group. Instead of the term tissue fibrinogen it would be better to describe this protein simply as tissue globulin, since it does not possess any direct relation to the formation of fibrin. It is not precipitated from its solutions by saturation with sodium chloride, but is thrown down by half saturation with ammonium sulphate. The precipitate in the latter case when redissolved in water exhibits the thromboplastic action of the original extract. It is evident also from the above facts that the destruction of thromboplastic activity by heating in saline and aqueous extracts of the tissues is due simply to the coagulation of this protein which carries down with it the active phosphatid material; the latter is not injured by the boiling. The contradiction between those who claim that the thromboplastic substance is thermolabile and those who insist that it is thermostable may therefore be reconciled.

The former are correct so far as the aqueous and saline extracts of the tissues are concerned, and the latter are correct in the case of the alcohol-ether extracts. The phosphatid body in aqueous solution is entirely thermostable, but in combination with tissue globulin it is precipitated out of solution by heat or other agencies which cause a coagulation of the protein, although itself not injured by the process, since it may be extracted readily from the coagulum by ether.

The Action of the Thromboplastic Substance.

That the thromboplastic action of the phosphatid (kephalin) solutions is due to a neutralization of antithrombin can be shown by direct experiments of the following character. In one set of experiments the thromboplastic solution was prepared as follows. The dried brain was extracted with ether. The ethereal solution was evaporated to dryness. The residue was extracted thoroughly with acetone to remove the cholesterin and fats and again dried at a low temperature. It was then dissolved in ether, and the ethereal solution was precipitated by the addition of alcohol. The precipitate and filtrate both exhibited thromboplastic action. The experiments were carried out with the filtrate, which was evaporated to dryness and then treated with water. The action of the opalescent solution obtained was tested upon hirudin and upon the antithrombin contained in peptone plasma. The hirudin solution was made by dissolving 0.1 gm. of commercial hirudin in 200 c.c. of a 0.9 per cent solution of sodium chloride, giving an approximate concentration of 1 to 20,000. The antithrombin of the peptone plasma was prepared by dissolving one of the watch crystals containing the dried plasma in 4 c.c. of a 0.9 per cent solution of sodium chloride, filtering and heating to 60° C. to precipitate the fibrinogen. The filtrate from this precipitate contained the antithrombin.

The idea of the experiments was to mix certain amounts of the thromboplastic solution with certain amounts of the hirudin or antithrombin solution and allow these mixtures to stand for a specified time, in one case for thirty minutes and in another for five hours. The action of these mixtures was then compared with similar mixtures in which the thromboplastic solution had been replaced by water and which had been allowed to stand for the same time. The comparisons were made by adding the mixtures in suitable amounts to a series of thrombin and plasma mixtures whose time of coagulation was known (three to eight minutes) and in which the amounts of thrombin were varied.

The plasma was used in place of a solution of fibrinogen and consisted of oxalated and centrifugalized dog's blood from which the excess of oxalate had been removed by dialysis. The clear plasma was then dried at low temperature in watch crystals and kept as a stock material of uniform properties. The results of the first series of observations may be stated as follows:

EXPERIMENTS WITH HIRUDIN. STAND OF THIRTY MINUTES.

I. Hirudin solution (1–20000) plus equal volume of water (Mixture A).

II. Hirudin solution (1–20000) plus equal volume of thromboplastic solution (Mixture B).

Control series.— 1. Mixture A, one drop; thrombin solution, three drops. Allowed to stand fifteen minutes, then added thirteen drops of plasma. No clot after twenty-four hours.

2. Mixture A, one drop; thrombin solution, four drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. No clot after twenty-four hours.
3. Mixture A, one drop; thrombin solution, five drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted partially in five hours thirty minutes.
4. Mixture A, one drop; thrombin solution, six drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in thirty minutes.

Series with thromboplastic solution. — 1. Mixture B, one drop; thrombin solution, three drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in fifty-five minutes.

2. Mixture B, one drop; thrombin solution, four drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in thirty to thirty-five minutes.
3. Mixture B, one drop; thrombin solution, five drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in ten minutes.
4. Mixture B, one drop; thrombin solution, six drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in five to ten minutes.

EXPERIMENTS WITH ANTITHROMBIN FROM PEPTONE PLASMA. STAND OF THIRTY MINUTES.

I. Antithrombin solution plus equal volume of water (Mixture A).

II. Antithrombin solution plus equal volume of thromboplastic solution (Mixture B).

Control series. — 1. Mixture A, one drop; thrombin solution, three drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Small membranous clot after twenty-four hours.

2. Mixture A, one drop; thrombin solution, four drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Beginning clot after six hours, fully clotted next morning (twenty hours).

3. Mixture A, one drop; thrombin solution, five drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted between one hour fifty-five minutes and two hours five minutes.

4. Mixture A, one drop; thrombin solution, six drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Beginning clot in sixty-five minutes, complete at one hour twenty minutes.

Series with thromboplastic solution. — 1. Mixture B, one drop; thrombin solution, three drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in sixty minutes.

2. Mixture B, one drop; thrombin solution, four drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in thirty-five minutes.

3. Mixture B, one drop; thrombin solution, five drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in fifteen minutes.

4. Mixture B, one drop; thrombin solution, six drops. Allowed to stand fifteen minutes, then thirteen drops of plasma. Clotted in ten to fifteen minutes.

Exactly similar results were obtained in a series made with the same solutions and in which the only variation introduced was that the mixtures of antithrombin and thromboplastic substance were allowed to stand five hours instead of thirty minutes before being tested upon the solutions of thrombin and plasma. The results of the second series showed that the neutralizing action of the kephalin solutions was as complete after thirty minutes as at the end of five hours. In a third series of experiments the thromboplastic solution was prepared from the residue of an ethereal extract of the brain after previous extraction with cold alcohol alone. Its action was tested upon the antithrombin of a peptone plasma by two different procedures: first, a solution in saline of the unheated peptone plasma was mixed with an equal volume of the thromboplastic solution, and after standing ten minutes but before clotting had occurred it was heated to 60° C. to precipitate

the fibrinogen. The filtrate (Mixture B) was tested for its antithrombic power in comparison with a control mixture (Mixture A) treated in the same way except that water was used in place of the thromboplastic solution.

I. Control with water (Mixture A).

1. Mixture A, one drop; thrombin solution, three drops. Allowed to stand fifteen minutes, then plasma thirteen drops. No clot at end of two hours; found clotted next morning (twenty hours).
2. Mixture A, one drop; thrombin solution, four drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted in one hundred minutes.
3. Mixture A, one drop; thrombin solution, five drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted in twenty-six minutes.
4. Mixture A, one drop; thrombin solution, six drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted in ten minutes.

II. With thromboplastic solution (Mixture B).

1. Mixture B, one drop; thrombin solution, three drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted in sixty-five to seventy minutes.
2. Mixture B, one drop; thrombin solution, four drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted in thirty-five minutes.
3. Mixture B, one drop; thrombin solution, five drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted in fifteen minutes.
4. Mixture B, one drop; thrombin solution, six drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted in ten minutes.

In the second procedure the saline solution of peptone plasma was heated to 60° C. to precipitate the fibrinogen, and the filtrate containing the antithrombin was divided into two parts. The first portion was mixed with an equal volume of thromboplastic solution (Mixture B) and the second with an equal volume of water (Mixture A). The two

mixtures were allowed to stand for twenty-five minutes and were then tested for their antithrombic power.

I. The control mixture (Mixture A).

1. Mixture A, one drop; thrombin solution, three drops. Allowed to stand fifteen minutes, then plasma thirteen drops. No sign of clotting after two hours; imperfect clot after twenty-four hours.
2. Mixture A, one drop; thrombin solution, four drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Beginning clot in two hours; found clotted in morning (twenty hours).
3. Mixture A, one drop; thrombin solution, five drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Beginning clot at fifty minutes, nearly complete at sixty-five minutes.
4. Mixture A, one drop; thrombin solution, six drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted in twenty minutes.

II. The thromboplastic mixture (Mixture B).

1. Mixture B, one drop; thrombin solution, three drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Beginning clot at seventy minutes, solid clot at one hundred five minutes.
2. Mixture B, one drop; thrombin solution, four drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Solid clot between forty and fifty-five minutes.
3. Mixture B, one drop; thrombin solution, five drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted between twenty-five and thirty minutes.
4. Mixture B, one drop; thrombin solution, six drops. Allowed to stand fifteen minutes, then plasma thirteen drops. Clotted at fifteen minutes.

These experiments all seem to give direct and conclusive proof that the thromboplastic solutions of brain phosphatid (kephalin) favor coagulation, in liquids containing antithrombin, by neutralizing the action of the antithrombin. So far as the author can see the only objection that can be made to this conclusion rests upon the hypothesis that the thromboplastic substance instead of neutralizing the antithrombin may have caused the production of more thrombin, by activation of prothrombin, in the plasma used for the coagulation test.

This objection is made invalid by the fact that the thromboplastic solutions added alone to the plasma used caused no coagulation, hence they had no power to convert prothrombin to thrombin. The further intrinsically improbable suggestion that the thromboplastic substance in some way may have augmented the supply of prothrombin in the plasma is also irrelevant. The plasmas used for the coagulation tests were oxalated plasmas, subsequently dialyzed to remove excess of oxalate, hence they were calcium free, and granting that in some way their supply of prothrombin may have been increased there was no chance for this substance to become activated to thrombin in a calcium-free mixture. It would have been possible of course in these experiments to have used a solution of fibrinogen instead of the oxalated plasma, but this did not seem to be necessary, and on the other hand it was very convenient to use the oxalated plasma, since, as described above, it could be kept on hand as a stock material of uniform composition. It is not possible to keep fibrinogen solutions by drying them down, as in the case of the plasma, nor is it possible to prepare from time to time fresh fibrinogen solutions of exactly the same concentrations.

Additional Properties of the Thromboplastic Phosphatid.

As prepared from the dried brain the thromboplastic phosphatid, after removal of fats, cholesterin, and lecithin, dissolves with some readiness when rubbed up with water, giving an opalescent solution free from visible or filtrable suspensions. From such a solution the material may be precipitated by the addition of small quantities of the neutral salts or the salts of the alkaline earths. For this reason it is advisable in making these thromboplastic solutions to use water instead of a 0.9 per cent solution of sodium chloride, since in the latter only a fine suspension may be obtained.

The aqueous solution of the phosphatid may be boiled without injuring its thromboplastic activity, but, on the contrary, it gradually deteriorates on standing. In one case, for example, an active solution of this kind was tested from time to time upon solutions of peptone plasma. When freshly made, this solution, added in equal volume, caused the peptone plasma (diluted once with water) to clot in ten to fifteen minutes. For three or four days this action was maintained with

but little visible variation, but at the end of eleven days its action had nearly disappeared. Tested upon specimens of the same plasma, there was no sign of coagulation after five hours and only an imperfect clot after twenty-four hours. The solution itself was unchanged in appearance. This result shows that a slow change of some kind gradually alters the phosphatid so as to destroy its thromboplastic power. It may also be used to refute the theory which has been proposed from time to time, especially by Nolf, that the thromboplastic action of such solutions is a physical reaction dependent upon the condition of fine suspension. In this case the physical characteristics of the solution had undergone no visible change. Indeed in many ways throughout these experiments it has been shown that the turbidity or state of suspension or opacity of the phosphatid solutions has nothing to do with their thromboplastic action. Similar solutions of egg lecithin, fine fat emulsions, etc., are entirely without action of this kind. The thromboplastic action of the tissue phosphatid (kephalin) is of a chemical rather than a physical nature.

Summary.

1. A method is described for preparing a stock material of dried peptone plasma to be used in testing the thromboplastic activity of tissue extracts.

2. Dilution with water diminishes markedly the effect of the antithrombin contained in peptone plasmas, while dilution with saline solutions (0.9 per cent sodium chloride) has no such effect.

3. Ethereal solutions of dried brain or thymus gland contain a phosphatid, corresponding in its properties to kephalin, which has marked thromboplastic activity.

4. Lecithin obtained from the egg yolk has no thromboplastic action, but the yolk contains a small amount of a phosphatid, corresponding to kephalin, which exhibits thromboplastic power.

5. In saline or aqueous extracts of the dried tissues (thymus, brain) the active phosphatid is combined with a protein having a low temperature of heat coagulation, 60° C. Heating such solutions or adding acid (acetic) precipitates the protein together with the active phosphatid. The phosphatid may be extracted from these precipitates with ether, and in aqueous solution is not affected by boiling. Aqueous

or saline extracts of the tissues are apparently thermolabile because the active substance is carried down with the protein coagulum.

6. The phosphatid contained in the ethereal extracts of the tissues exerts its thromboplastic effect upon blood plasmas by neutralizing the action of the contained antithrombin.

7. Aqueous solutions of the active phosphatid are precipitated by the addition of neutral salts, or salts of the alkaline earths. The thromboplastic activity of the aqueous solutions disappears slowly on standing.

THE UNIVERSITY PRESS, CAMBRIDGE, U.S.A.